in Opa1c.984G>A cells. Thus, transient fusions are spared and seem to be sufficient to support mitochondrial metabolism in Opa1c.984G>A fibroblasts, exposing a mechanism that may contribute to the lack of severe clinical symptoms in ADOA associated with some Opa1 mutations.

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Mitochondrial Ca^{2+} Uptake; Regulation by Ca^{2+}, Inhibition by Minocyclin

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Mitochondrial Ca^{2+} uptake is mediated by the low-affinity Ca^{2+} uniporter (MCU) that is controlled allosterically by Ca^{2+}. The Ca^{2+} effect might be mediated by MICU1, a recently identified obligatory component of MCU. Ca^{2+} induces sensitization of MCU but some reports claimed Ca^{2+}-induced desensitization. Here, we compared recovery kinetics of cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_c$) elevations following addition of varying Ca^{2+} doses in suspensions of permeabilized RBL-2H3 cells. The recovery rates (reflecting mitochondrial Ca^{2+} uptake) progressively increased with the Ca^{2+} dose. The $[Ca^{2+}]_c$ recovery steady state was similar after 7.5-50uM $CaCl_2$ pulses, but was reached faster after the larger Ca^{2+} doses. Thus, a pre-exposure to high $[Ca^{2+}]_c$ enhanced the permeability of MCU in a prolonged manner resulting in accelerated mitochondrial Ca^{2+} uptake even at low $[Ca^{2+}]_c$. We are currently investigating the molecular mechanism underlying the effect of Ca^{2+}.

The most specific pharmacological inhibitor of MCU is Ru360 that has limited plasma membrane permeability. Minocyclin, an anti-inflammatory drug has been suggested to exert its cytoprotective and anti-apoptotic effect via interfering with mitochondrial Ca^{2+} uptake, based on studies of isolated mitochondria. However, minocyclin has also been shown to act as a Ca^{2+}-dependent protonophore. We found that minocyclin inhibits the rapid phase of IP_3-induced mitochondrial matrix $[Ca^{2+}]$ response at concentrations of 80-120uM in permeabilized RBL-2H3 cells almost as effectively as Ru360 (80% vs. 92% inhibition, respectively). In minocyclin-pretreated cells, the IP_3-induced Ca^{2+} release caused only slow and small mitochondrial depolarization, while bulk $[Ca^{2+}]_c$ elevation by addition of large amounts of Ca^{2+} elicited almost complete depolarization. These data suggest that the protonophore action of minocyclin requires relatively large $[Ca^{2+}]_c$. Thus, minocyclin seems to be an effective inhibitor of mitochondrial Ca^{2+} uptake sites during physiological $[Ca^{2+}]_c$ signals and exerts its protonophore effect under conditions of large global Ca^{2+} exposure.

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Regional Specialization of Mitochondrial Ca^{2+} Signaling in Cardiac Cells

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Excitation-contraction coupling in cardiac cells is controlled mainly by I_{Ca}-gated release of Ca^{2+} from the sarcoplasmic reticulum (SR), but is also thought to be modulated by mitochondrial Ca^{+} signaling. Using field-stimulated rat ventricular cardiomyocytes, we have previously found that mitochondria that were primed by rapid beating or caffeine-induced SR-Ca^{2+} release subsequently were capable of rapid release of Ca^{2+} when stimulated by shear forces (Belmonte and Morad, 2008, J. Physiol. **586**: 1379). To elucidate this finding we measured mitochondrial Ca^{2+} using Mitycam. The adenoviral Mitycam probe was originally created using the inverse pericam mutant M13-EYFP (V68L/Q69K)(145-238) and EYFP(V168L/Q69K)(1-144)-CaM, fused to the subunit VIII of human cytochrome *c* oxidase mitochondrial-targeting signal (Kettlewell *et al*., 2009, J Mol Cell Cardiol, **46**:891). Short-time cultured adult feline cardiomyocytes were infected with the mitycam adenovirus at MOI of 200 virus particles per cell. After 3-4 days we measured the Mitycam fluorescence under voltage-clamp conditions using whole-cell fluorometry and 2-D confocal imaging. Supporting information was obtained using TMRE to assess mitochondrial depolarization and NCX-currents to assess cytosolic Ca^{2+} transients. The whole-cell Mitycam measurements suggested transfer of Ca^{2+} from the SR to the mitochondria during and following voltage-clamp depolarizations and caffeine-induced SR Ca^{2+} release. In confocal measurements the distribution of Mitycam-fluorescence showed longitudinal streaks with sarcomeric banding resembling the TMRE staining and a characteristic nuclear band (NB) of enhanced fluorescence that typically connected and extended from the two nuclei. Depolarization, caffeine, mechanical stimulation, and FCCP produced changes in Mitycam fluorescence that often resulted in redistribution between NB, general mitochondrial, and subsarcolemmal mitochondrial compartments. Similarly, injection of FCCP caused local decline in TMRE fluorescence (mitochondrial depolarization), but strongly enhanced subsarcolemmal fluorescence. These findings suggest that mitochondria in different regions of cardiac cells play different roles in Ca^{2+} signaling. NIH HL16152.

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Quantification of Mitochondrial Calcium Dynamic Changes During Voltage-Induced Calcium Release in Mammalian Skeletal Muscle

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Mitochondrial Ca^{2+} uptake regulates mitochondrial metabolism and synthesis of ATP to meet demands of muscle contraction. In a recent study, we found that mitochondrial Ca^{2+} uptake also plays a critical role in modifying rapid Ca^{2+} transients in skeletal muscle of amyotrophic lateral sclerosis transgenic mice (*Zhou 2010*). To better understand how mitochondria are involved in the control of Ca^{2+} transients in healthy and diseased conditions, we need to trace dynamic changes of Ca^{2+} inside mitochondria during contractile activation. *Rudolf* (*2004*) first demonstrated mitochondrial Ca^{2+} uptake in skeletal muscle during contraction using the cameleon YC2. The maximal ratio change, however, was not more than 0.4, and Ca^{2+} uptake was not quantified. We have now used the improved biosensor YC3.6 (*Nagai 2004*) with a dynamic range close to 6 (in *in-situ* calibration) to monitor changes of free $[Ca^{2+}]$ inside mitochondria during voltage clamp-induced Ca^{2+} release (VICR). We targeted YC3.6 to mitochondria by adding a mitochondrial signal sequence at 5' of the cDNA to obtain mt11-YC3.6. One week after FDB muscles of adult mice were transfected by electroporation, enzyme-isolated single FDB fibers expressing mt11-YC3.6 were patch-clamped. Following a depolarizing pulse, the cytosolic Ca^{2+} transient (monitored by x-rhod-1) and the change of Ca^{2+} inside mitochondria were simultaneously recorded in a confocal microscope. We found that the maximal ratio change of mt11-YC3.6 reached 3.25 following a membrane depolarization. The free $[Ca^{2+}]$ inside mitochondria during VICR was calculated using parameters from an *in-situ* calibration. $[Ca^{2+}]_{mito}$ increased to 200 nM during a 10 ms pulse and reached 3 μM during a 800 ms pulse. These results and similar measurements in progress will allow us to evaluate the uptake of Ca^{2+} by mitochondria during single twitches and tetanic contraction. Supported by MDA/NIAMS.

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Isoform-And Species-Dependent Sensitization of the IP3 Receptor by Superoxide Anion

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Multiple interactions have been described between reactive oxygen species and calcium signaling but the underlying mechanisms remain elusive. In human HepG2, rat RBL-2H3, and chicken DT40 cells, we observed cytoplasmic [Ca2+] ([Ca2+]c) spikes and frequency-modulated oscillations evoked by a superoxide anion donor, xanthine (X)+xanthine oxidase (XO), dose-dependently. The [Ca2+]c signal was also observed when extracellular Ca2+ was removed or mitochondria were uncoupled but was eliminated upon ER Ca2+ depletion by thapsigargin, indicating that superoxide stimulated ER Ca2+ mobilization. To test if superoxide affects the IP3 receptor (IP3R), we measured the IP3-induced Ca2+ mobilization in the presence or absence of X+XO in permeabilized cells. X+XO promoted the response to submaximal doses of IP3 but did not change the effect of maximal IP3, indicating sensitization of the IP3R by superoxide. To test the sensitivity of each IP3R isoform to superoxide we used DT40 cells lacking two out of three (DKO) or all IP3R isoforms (TKO). In response to X+XO, DKO expressing either type 1 (DKO1) or type 2 IP3Rs (DKO2) showed a [Ca2+]c signal, whereas DKO expressing type 3 IP3R (DKO3) or the TKO did not show a [Ca2+]c elevation. By contrast, IgM that stimulates IP3 formation, elicited a [Ca2+]c signal in every DKO and caused no [Ca2+]c increase in the TKO. These results indicate that each IP3R isoform could be activated by IP3 but only IP3R1 and IP3R2 are sensitized by superoxide. Further supporting this conclusion, X+XO facilitated the Ca2+ release evoked by submaximal IP3 in permeabilized DKO1 and DKO2 but was not effective in DKO3. Surprisingly, X+XO could also facilitate the effect of low IP3 in TKO transfected with rat IP3R3. We are currently studying if the molecular structure of IP3R3 might explain the isoform and species-dependent sensitization by superoxide.

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Resin-Assisted Capture Methods Show that *S*-Nitrosylation Exerts Cardioprotection During Ischemia/Reperfusion Injury by Directly Reducing Cysteine Oxidation

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Redox modifications play an important role in many cellular processes, including cell death. Ischemic preconditioning (IPC) has been shown to involve redox signaling and protein *S*-nitrosylation (SNO) is greatly increased following